

FINAL REPORT

For

Evaluation of Jet Fuel Induced Hearing Loss in Rats

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14. ABSTRACT The objective of the current work was to evaluate the potency of JP-8 jet fuel to enhance noise-induced hearing loss (NIHL) using inhalation exposure to fuel and simultaneous exposure to either continuous or intermittent noise exposure over a 28 day exposure period using both male and female Fischer 344 rats. In the initial study, male (n=5) and female (n=5) rats received inhalation exposure to JP-8 fuel for 6 hr/day, 5 days/week for 28 days at doses of 200 mg/m3, 750 mg/m3, and 1500 mg/m3. Parallel groups of rats also received non-damaging noise (constant octave band noise at 85 dBlin) in combination with the fuel, noise alone (75, 85, and 95 dB), fuel alone (200 mg/m3, 750 200 mg/m3, and 1500 mg/m3) , or no exposure to fuel or noise. Significant dose related impairment of auditory function measured by distortion product otoacoustic emissions (DPOAE) and compound action potential (CAP) threshold was seen in rats exposed to combined JP-8 plus noise exposure when JP-8 levels of 1500 mg/m3 were presented with trends toward impairment seen with 750 mg/m3 JP-8 + Noise . JP-8 alone had no effect on auditory function. In a subsequent study, male (n=5) and female (n=5) rats received 1000 mg/m3 JP-8 for 6 hr/day 5 days/week for 28 days with and without exposure to 102 dB octave band noise that was present for 15 min out of each hour (total noise duration 90 min). Comparisons were made to rats receiving only JP-8, only noise, and those receiving no experimental treatment. Pronounced impairment of auditory thresholds especially for high frequency tones were identified in the male rats receiving combined treatment.					
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ABSTRACT

The objective of the current work was to evaluate the potency of JP-8 jet fuel to enhance noise-induced hearing loss (NIHL) using inhalation exposure to fuel and simultaneous exposure to either continuous or intermittent noise exposure over a 28 day exposure period using both male and female Fischer 344 rats.

In the initial study, male (n=5) and female (n=5) rats received inhalation exposure to JP-8 fuel for 6 hr/day, 5 days/week for 28 days at doses of 200 mg/m³, 750 mg/m³, and 1500 mg/m³. Parallel groups of rats also received non-damaging noise (constant octave band noise at 85 dB_{lin}) in combination with the fuel, noise alone (75, 85, and 95 dB), fuel alone (200 mg/m³, 750 200 mg/m³, and 1500 mg/m³), or no exposure to fuel or noise. Significant dose related impairment of auditory function measured by distortion product otoacoustic emissions (DPOAE) and compound action potential (CAP) threshold was seen in rats exposed to combined JP-8 plus noise exposure when JP-8 levels of 1500 mg/m³ were presented with trends toward impairment seen with 750 mg/m³ JP-8 + Noise. JP-8 alone had no effect on auditory function.

In a subsequent study, male (n=5) and female (n=5) rats received 1000 mg/m³ JP-8 for 6 hr/day 5 days/week for 28 days with and without exposure to 102 dB octave band noise that was present for 15 min out of each hour (total noise duration 90 min). Comparisons were made to rats receiving only JP-8, only noise, and those receiving no experimental treatment. Pronounced impairment of auditory thresholds especially for high frequency tones were identified in the male rats receiving combined treatment.

INTRODUCTION

Laboratory investigations have identified a variety of chemicals of both occupational and environmental interest that are capable of producing hearing loss. The relevance of such data for human occupational exposure has been questioned at times because, in general, very high dose levels relative to permissible exposure levels (PELs) established for human occupational exposures and protracted exposure times are needed for ototoxicity to be observed. For example, toluene ototoxicity in rats was seen at exposure levels between 1000-2000 ppm over 3-5 days with 1300 ppm seen as a threshold dose for permanent hearing loss when exposures of 4 weeks are utilized (Crofton *et al.*, 1994, Johnson and Canlon, 1994, Pryor *et al.*, 1984, Rebert *et al.*, 1983, Sullivan *et al.*, 1988). Ethylbenzene ototoxicity has been observed at exposure doses of 300-400 ppm for 5 days (Cappaert *et al.*, 2000). And p-xylene ototoxicity occurs at exposure levels of 1800 ppm for 3 weeks (Maguin *et al.*, 2006). OSHA has set PELs for toluene at 200 ppm, and for xylenes and ethylbenzene at 100 ppm. The ACGIH has recommended TLVs of 50 ppm for toluene, and 100 ppm for xylenes and ethylbenzene. However, it has also been documented in laboratory animals that ototoxicity can be observed at more realistic exposure doses if noise is also present in the environment. This finding of an interaction between the effects of noise and chemical agents on hearing loss is particularly problematical given that in most instances occupational exposure levels are established based upon exposures to a single agent rather than to complex mixtures of agents. In only a few rare exceptions, for example, does the ACGIH recommend that auditory testing be pursued more aggressively if select chemicals are present in noisy environments (ACGIH, 2002).

This investigation was undertaken to determine the ototoxic potential of subchronic JP-8 jet fuel both by itself and in the presence of either continuous or interrupted noise. JP-8 is a traditional petroleum-derived fuel that is closely related to Jet A fuel used in commercial aviation. Both of these aviation fuels contain aromatic hydrocarbons (25% maximum). JP-8, designated as MIL-DTL-83133, has become the standard fuel used by the US armed services and by NATO. Several of the aromatic hydrocarbons contained in JP-8 fuel are known to be ototoxic based upon both epidemiological (Abbate et al., 1993; Morata, et al, 1997; Vrca et al. 1996, 1997; Sliwinska-Kowalska, et al. 2001, 2003; Fuente et al., 2009) as well as controlled laboratory studies (Campo et al., 1997, 2001; Cappaert et al., 1999, 2000, 2001a,b,; Crofton, 1994; Loquet, 1999; Pryor et al., 1983,1987; McWilliams et al., 2000; Lataye et al., 2003; Gagnaire and Langlais, 2005).

More to the point, there have been prior investigations both of the effects of jet fuel on hearing in occupational settings as well as short-term studies among laboratory subjects. Kaufman et al. (2005) studied a small sample of U.S. Air Force employees with occupational exposure to noise and jet fuels (JP-4 and JP-8) containing aromatic hydrocarbons and reported that jet fuel may increase hearing loss in a chronic exposure model (a larger odds ratio for hearing loss with 12 years of exposure than for 3 years of exposure). Moreover, the odds ratio associated with duration of fuel exposure exceeded that obtained for age. However, since all subjects did have a history of noise exposure it is not clear whether the fuel by itself might have produced some ototoxicity. There is a high probability for combined exposure to jet fuel and to noise in a wide range of occupations related to airplane operations.

Fechter, et al., (2007, 2010) reported that subacute exposures in rats to JP-8 jet fuel by itself had no effect on auditory function up to doses of 2000 mg/m³ assessed using either distortion product otoacoustic emissions (DPOAE) or pure tone auditory thresholds. The later was assessed by measuring the occurrence of a compound action potential (CAP). However, exposure to JP-8 did enhance the adverse effects of moderate noise exposure on DPOAE amplitude. Specifically, successive exposure first to JP-8 jet fuel (1000 mg/m³ for 4 hr/day x 5 days) followed on each of the five days by a 1 hr exposure to 100 dB_{lin} octave band noise (OBN) yielded a persistent reduction in the DPOAE. The noise exposure alone produced minimal impairments on this measure of outer hair cell (OHC) function.

The current study used a more appropriate design for evaluating the joint effects of JP-8 and noise exposure in that noise and fuel exposures occurred simultaneously over a longer time period each day and over 28 day duration (5 days/week for 4 weeks). This study required the identification of a noise exposure protocol that yielded the lowest observed adverse effect (LOAEL) on hearing, a dose response study to identify a LOAEL and no observed adverse effect level (NOAEL) for JP-8 jet fuel by itself, and the characterization of JP-8 exposure able to increase susceptibility to NIHL. Once these objectives were met using a continuous noise exposure paradigm, an additional study was undertaken to determine the efficacy of JP-8 to promote NIHL induced by an intermittent noise exposure. Intermittent noise is a far more common workplace experience than is continuous noise over the course of a work day.

METHODS AND MATERIAL

Subjects

For all studies, Fisher 344 male and female rats obtained from Charles River Laboratories (Wilmington, MA) were employed as the test animals. The rats were purchased at approximately 6 weeks of age and initially were housed at WPAFB, Dayton OH. Seven days following their arrival, the auditory function of the rats was assessed by the distortion product otoacoustic emissions (DPOAE) test in order to equate auditory function across all groups (described below). The rats then received their assigned noise and JP-8 exposure. Three days following the conclusion of experimental exposures, the rats were transported by temperature controlled vans and commercial airplane to the Jerry Pettis Memorial VA Medical Center in Loma Linda, CA where they received extensive auditory testing and ultimately were euthanized and cochleae harvested for assessment of inner ear pathology. The subjects were housed in plastic cages with free access to food and water. Temperature was maintained at $21 \pm 1^{\circ}\text{C}$ and fluorescent lights were on from 6:30 A.M. to 6:30 P.M. All procedures used were approved by the Institutional Animal Care and Use Committees (IACUCs) both at WPAFB and at the LLVAMC. All exposures and testing were performed during the daytime.

Exposure Procedures

Because of obvious limitations posed by the number of inhalation chambers available, a series of studies was conducted in which (a) an appropriate continuous noise exposure level was determined (see below), (b) a dose response study for the effect of JP-8 alone on auditory function was conducted and, (c) a study in which the effects of

simultaneous continuous noise and JP-8 exposure were determined. Finally, a study of the interaction of JP-8 jet fuel and intermittent noise exposure was undertaken. Table 1 summarizes the exposure treatments used in each experiment and Figure 1 depicts the jet fuel generation system and an exposure chamber.

Table 1. Summary of Treatments

	males (n)	females (n)
Experiment		
<u>Noise dose response</u>		
75 dB OBN 6hrs/day, 5 days/week for 28days	5	5
85 dB OBN 6hrs/day, 5 days/week for 28days	5	5
95 dB OBN 6hrs/day, 5 days/week for 28days	5	5
Controls	5	5
<u>JP-8 vs JP-8 + continuous noise</u>		
JP-8 200mg/m3 6hrs/day, 5 days/week for 28 days	5	5
JP-8 750mg/m3 6hrs/day, 5 days/week for 28 days	5	5
JP-8 1500mg/m3 6hrs/day, 5 days/week for 28 days	5	5
Controls	5	5
JP-8 200mg/m3 + 85 dB OBN 6hrs/day, 5 days/week for 28 days	5	5
JP-8 750mg/m3 + 85 dB OBN 6hrs/day, 5 days/week for 28 days	5	5
JP-8 1500mg/m3 + 85 dB OBN 6hrs/day, 5 days/week for 28 days	5	5
Controls	5	5
<u>JP-8 + intermittent noise</u>		
JP-8 1000mg/m3 6hrs/day, 5 days/week for 28 days	5	5
JP-8 1000mg/m3 6hrs/day, 5 days/week for 28 days + 102 dB intermittent OBN 90min/day total	5	5
102 dB intermittent OBN totaling 90min/day, 5 days/week for 28 days	5	5
Controls	5	5

Jet fuel

Three different doses of jet fuel were used in the initial study (200, 750 and 1500 mg/m³ - total hydrocarbon levels) which bracketed the dose previously shown to promote NIHL using a nose only exposure for 5 days (Fechter et al., 2007, 2010). For comparison, the permissible concentration for JP-8 in the workplace is 200 mg/m³ based on an 8 h time weighted average (TWA). Half of the rats receiving jet fuel also received noise exposure as described below while the remaining rats received only the JP-8 exposure in order to evaluate its ototoxicity.

The fuel was supplied from a stock maintained by the Fuels Branch (AFRL/RZPF) at Wright-Patterson Air Force Base (Dayton, OH). It consisted of a blend of jet fuel obtained from various refineries to which was added the JP-8 fuel additive package consisting of diethylene glycol monomethyl ester (0.1 vol/vol %) to inhibit ice formation, and both proprietary static (2 mg/L) and corrosion (15 mg/L) inhibitors. A single lot of fuel was used to complete all of the studies described here.

The fuel generation system for the high concentration of 1500 mg/m³ is shown in Figure 1.A. Jet fuel was pumped from a reservoir using a FMI Model QG20 pump with a Q1CKC pump head (FMI Inc., Syosset NY) into the fuel input port of a Sonimist ultrasonic spray nozzle (Misonix Inc., Model HSS600-2, Farmingdale, NY 11735). An air line set to 40 pounds per square inch (psi) pressure was attached to the side arm of the Sonomist. At this pressure the spray nozzle developed an air flow of approximately 20 liters per minute (lpm) through the nebulizer. This air flow coupled with the nebulizer nozzle design created an ultrasonic whistle which aerosolized the droplets of jet fuel being formed at the end of the nozzle and acted as a carrier for the jet fuel into the

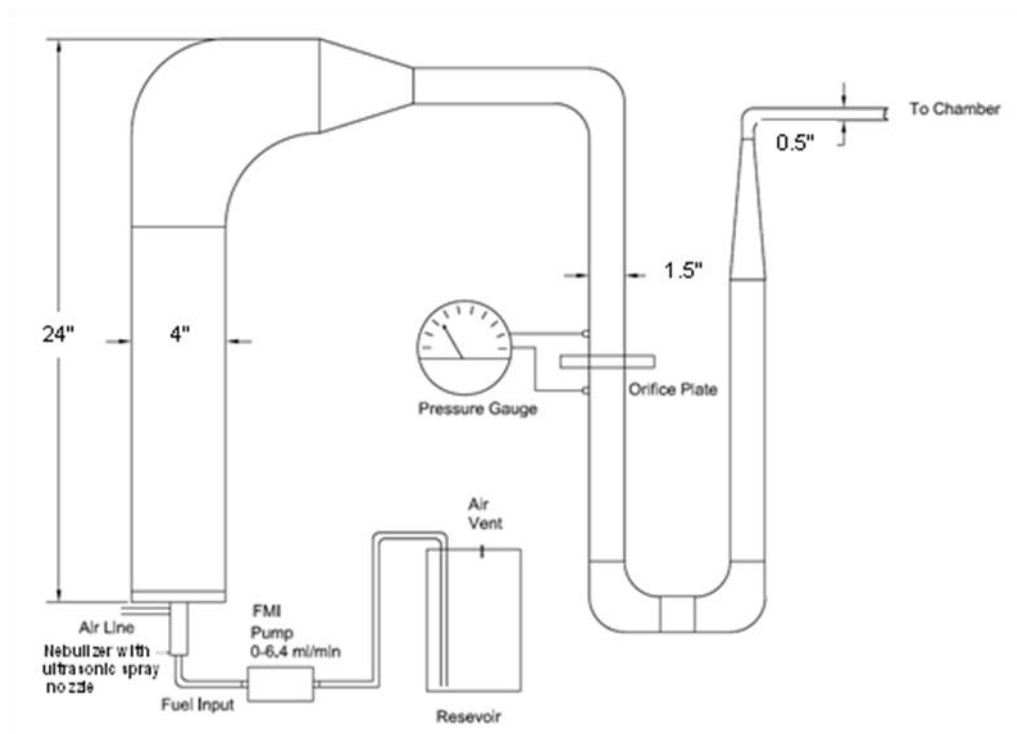


Figure 1.A. Schematic representation of JP-8 jet fuel generation system for inhalation exposure.

generation system. A two foot length of four inch PVC pipe contained the spray pattern. The pipe was initially reduced in size to accept an orifice plate which can be used to measure flow rate by the pressure drop across the plate. However, no orifice plate was used in the line for the current system. The pipe diameter was reduced one final time to three quarters inch and the aerosolized jet fuel was transported to the chamber where it was injected as a counter current into the main chamber flow. Two drain ports were used to remove residual jet fuel which accumulated after a day's exposure. To achieve the

1500 mg/m³ concentration, the high generation system used a HSS600-2 nebulizer, which has greater throughput and did not develop problems with fuel accumulation around the nebulizer. During exposure a small amount of excess fuel accumulated in the system at the drain ports. Adding auxiliary air kept the jet fuel accumulation to a minimum and prevented interference with flow.

The mid and low concentration generation systems used a Sonomist HSS600-1 nebulizer, no orifice plate and a 0.5 inch line to the chamber (in place of the 0.75 inch line). To eliminate problems with occasional nebulizer malfunction due to jet fuel accumulation around the nebulizer the 4 inch pipe was inverted so the nebulizer aimed down rather than up as in Figure 1.A. The high generation system could not be inverted due to differences in the parts used to assemble that system. The mid concentration system was still accumulating too much jet fuel in the lower parts where the drain ports were added so auxiliary air was added to the mid range system as well which eliminated jet fuel accumulation.

The rats were exposed to JP-8 using a whole body exposure system consisting of whole body 690 liter toxic hazard research units (THRU) chambers (Figure 1.B.). Each chambers was operated with a total flow of 180 liters per minute (lpm) consisting of the combination of jet fuel generator input and the main air flow. The main air flow was supplied by two Spencer vortex blowers (model VB030SB-012) one provided input air and one handled exhaust flow. The exhaust air flow was adjusted to maintain a slightly negative, one to two inches of water, sub-ambient pressure inside the chamber as measured with a magnehelic pressure gauge (Dwyer Instruments, Champlain, NY) attached to the upper plenum of the chamber. Airflow through the chambers was

controlled with mechanical valves that were adjusted to obtain the desired flow rate. Flow rate was monitored on the input side of the chamber using a Hastings (Teledyne-Hastings, model LSD58D, Hampton VA) laminar flow unit, and the signal was monitored using a Hastings (Model 40) monitor. The back of the chamber has 9 ports which can be used for various sampling devices. Attached to one port was a Nicolet (Thermo Scientific, Model IS10) Fourier Transform Infrared Spectrophotometer (FTIR) equipped with either a 2 meter path length gas cell for high concentrations or a 10 meter

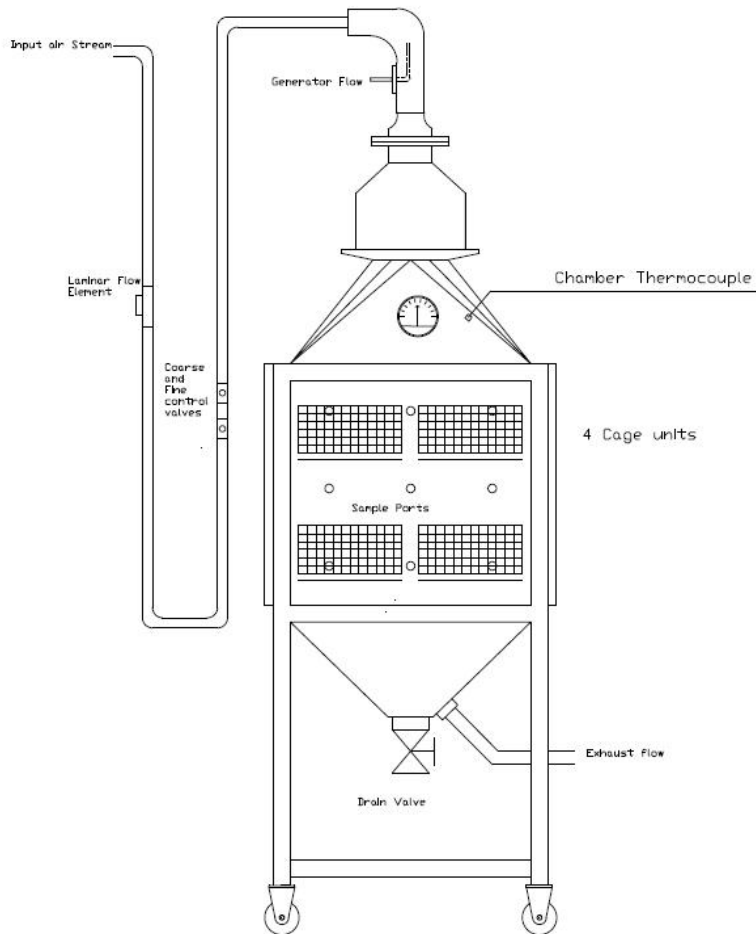


Figure 1.B. Schematic representation of a whole body exposure chamber.

path length gas cell for lower concentrations. Prior to entering the FTIR, the aerosol portion of the sample was removed using a small HEPA filter. Sampling by the FTIR was controlled using a macro on a computer which averaged every 10 spectrums collected, displayed the average concentration of jet fuel on the screen as well as saved the data to a file. The system was programmed to collect and save one sample per minute for the entire 6 hour exposure period.

Noise exposure

The noise exposure selected was designed to produce a just observable permanent impairment in auditory function, but one small enough such that additive or potentiating effects of chemical exposure could also be detected (e.g., Pouyatos et al., 2005; Rao and Fechter, 2000). The noise level used was an octave band (OBN) centered at 8 kHz so as to yield cochlear injury at frequencies within the most sensitive portion of the rat's audiogram (approximately 8-20 kHz).

Current OSHA standards have established a PEL for noise of 90 dB using the A weighting scale for an 8 h TWA with an action level of 85 dB(A) at which point specific measures must be adopted to limit noise exposure (29 CFR 1910.95, 1998). Audiograms are an annual requirement. A 5 dB exchange rate is utilized for intermittent noise and for noise that does not persist for 8 hr. Based upon this rule, the equivalent human PEL for a 4-h time period would be 95 dB (A) and, for 1 h, 105 dB (A). In the initial noise study, exposure levels of 75, 85, and 95 dB were employed for time periods of 6 hr. These noise levels are equivalent to approximately 72, 82, and 92 dB (A) on an 8 hr exposure basis.

Thus the noise employed in these studies would bracket the exposure limit permitted by OSHA for workplace exposures with only the highest exposure level exceeding the human occupational PEL. Computer software installed on a laptop computer was used to generate a pure and precisely filtered white noise file. A high pass filter with a 48 dB per octave roll-off was applied within the software to attenuate frequencies below 5.6 kHz, followed by a low pass filter with the same roll-off value to attenuate frequencies above 11.3 kHz. The filter produced a finished file to one octave band wide, centered at 8 kHz. The filtered file was then played through electrodynamic shakers that induced vibration from the outside in the metal plenums at the bottom of each exposure chamber. During exposures the sound intensity was measured inside the chambers at a central reference point using a Spectral Dynamics Puma data acquisition system (Spectral Dynamics, San Jose, California). The system had four active input channels for monitoring and recording real-time sound levels in four chambers simultaneously. A 20-foot coaxial cable was connected to each of the output channels and a PCB Model 378B20 1.27 cm (0.5 inch) random incidence microphone assembly was connected to the other end of each cable. A 1.27 cm (0.5 inch) inside diameter PVC pipe was installed through the center port on the rear of each chamber so the microphone could be positioned at the central reference point. Sound pressure measurements for chamber characterization were made using a Larson Davis Model 831 sound level meter with a 6.1 meter (20 foot) extension cable and microphone preamplifier. Distribution of sound pressure levels across 10 chamber exposure points were well controlled within ± 1.5 dB. Stability at the central reference point was well controlled over 6-hour runs within ± 1

dB. Following completion of an initial noise intensity study conducted with the purpose of assessing a NOAEL for noise alone and a similar JP-8 dose response study, two studies were completed in which the rats were assigned to receive noise exposure along with jet fuel inhalation while the remaining subjects received no experimental exposure (control).

Auditory Assessment

Outer hair cell (OHC) functional assessment: DPOAE

Outer hair cell (OHC) function was assessed in subjects prior to any other experimental manipulation as a means of equating auditory function across treatment groups in a non-invasive manner. The DPOAE test relies upon the finding that the intact cochlea is able to generate measurable sound energy when stimulated with two simultaneous tones known as “primary tones” and designated as frequencies “ f_1 ” and “ f_2 .” Because the sound energy generated by the cochlea consists of different frequencies than the “primary tones” they are spoken of as “distortion products.” A particularly robust distortion product is the cubic distortion product which is defined algebraically as $2 f_1 - f_2$. If the ratio of f_1 / f_2 is kept constant as the frequency of f_2 is swept along the subject’s audiometric range, it is possible to detect impairment of the hair cells as a drop in DPOAE amplitude. In these experiments, the ratio of f_1 / f_2 was maintained at 1.25 and the f_2 frequency was swept from 3.197 to 19.401 in the initial screening for auditory function. A more extensive evaluation of DPOAE amplitude was undertaken in all post exposure assessments. Here the f_2 frequency was swept from 3.1 to

63 kHz in 0.1-octave increments. Tone intensities were set at 55 dB for f_1 and 35 dB for f_2 . This difference in tone intensity was selected to maximize the amplitude of the DPOAE (Whitehead et al., 1995). The f_1 and f_2 primaries were presented through two separate Realistic dual radial horn tweeters (Radio Shack, Tandy Corp., Ft Worth, TX). The tones were delivered to the outer ear canal through a probe that also contained an emissions microphone assembly (Etymotic Research, ER-10B+, Elk Grove Village, IL). The tones were sampled, synchronously averaged, and Fourier analyzed for geometric mean frequencies. Delivery of the primary test tones and computation of the $2 f_1 - f_2$ distortion product amplitude were accomplished by a digital sound processing (DSP) board (National Instruments model PCI-4461, Austin, TX) controlled by a dedicated program written using LabVIEW version 7.1 (National Instruments, Austin, TX). The related noise floors were estimated by averaging the levels of the ear-canal sound pressure for the two fast Fourier transform frequency bins below the DPOAE frequency (i.e., for 3.75 Hz below the DPOAE). A hard-walled cavity that approximated the size of the rat outer-ear canal was used to calibrate the tonal stimuli. For both stimulus protocols, DPOAEs were considered to be present when they were at least 3 dB above the noise floor.

DPOAE testing was accomplished in a single walled audiometric booth while rats were lightly anaesthetized with ketamine (44 mg/kg) and dexdomitor (0.25 mg/kg) injected im. Normal body temperature was maintained using a dc heating unit built into the table supporting the rat. To assess the effects of noise intensity alone on auditory function in the initial study, DPOAE amplitudes were assessed at only 1 post exposure time point, 4 weeks, so that permanent impairment could be assessed. In subsequent

studies, each subject was tested 10 days after the end of the experimental treatment, and, again, 4 weeks post-exposure. Each DPOAE test required approximately 3 min to perform. The rats subsequently received a dose of atipamezole (0.1mg/rat) to reverse the anaesthesia.

Audiometric threshold assessment: CAP

In contrast to the repeated assessment of OHC function by the DPOAE method, assessment of auditory threshold, a marker of neural activity in the auditory branch of the eighth cranial nerve, requires non-survival surgery. Threshold assessment was performed 4 weeks following the end of all experimental exposures by recording the CAP from the round window for pure tones between 2 and 40 kHz in approximately $\frac{1}{2}$ octave steps. The CAP is a marker of synchronous auditory nerve action potentials elicited by pure tone stimuli. Auditory thresholds were assessed in a double walled audiometric booth. Preparation of subjects for CAP assessment required non-survival surgical procedures performed under anaesthesia (75 mg/kg ketamine and 0.5mg/kg dexdomitor). The auditory bulla was opened via a ventrolateral approach to allow the placement of a fine (od 0.1 mm) Teflon-coated silver wire electrode (A-M Systems, Inc., Carlsborg, WA) onto the round window. A silver chloride reference electrode was inserted into neck musculature. The cochlea was warmed using a low voltage high-intensity lamp. Tonal stimuli were generated and shaped using a SoundMax Integrated Digital Audio board. A dedicated program running within LabVIEW 7.1 (National Instruments, Austin, TX) was used to control stimulus intensity, frequency, and timing. Each pure tone stimulus consisted of a 10 msec burst with 1 msec onset and offset ramps. Tones were presented at a frequency of 9.7/sec. The computer program allowed tones to be augmented in 1 dB

intensity steps until a discernable CAP was identified on a digital oscilloscope by the experimenter. The CAP signals evoked by pure tones were amplified x1000 between 0.1 and 1.0 kHz with a Grass A.C. preamplifier (Model P15, W. Warwick, RI). The sound level necessary to generate a visually detectable CAP response averaged over four sweeps on a digital oscilloscope (approximate response amplitude of 1 mV measured as the output of the preamplifier) was identified. Identification of the N₁ response was based upon shape of the response as well as its temporal relationship to the onset of the tonal stimulus. The CAP threshold was defined as the highest stimulus intensity at which the N₁ response was no longer observed against the noise background.

Histopathology

In addition to the functional testing described above, subjects were euthanized at the conclusion of testing and cochleae were harvested for evaluation of hair cell death. Immediately after CAP measurements, rats were decapitated and the cochleae harvested. Within 2 min, the cochleae were fixed by perilymphatic perfusion with 1 ml of a trialdehyde fixative (3% glutaraldehyde, 2% formaldehyde, 1% acrolein and 2.5% DMSO in phosphate buffered saline pH7.4). Following the primary 24h-fixation, the tissue was first washed with 0.1M phosphate buffered saline, post-fixed with 2% osmium tetroxide in water for 2 h, and finally washed again with 0.1M phosphate buffered saline. The organ of Corti was dissected in 70% ethanol and mounted in glycerin to allow counting of the hair cells. Cells were counted as present either when the stereocilia, the cuticular plate or the cell nucleus could be visualized. No attempt was made to assess the degree of possible cellular damage to surviving cells. The frequency-place map

established by Muller (1991) was used to superimpose the frequency coordinates on the length coordinates of the organ of Corti. This “map” reflects the fact that the cochlea is organized in a tonotopic fashion with high frequency sound producing maximum stimulation of cells in the base, and low frequency sound in the apex. A cochleogram showing the percentage of hair cell loss as a function of distance from the apex of the cochlea was plotted for each animal. The results were averaged within each group of subjects for comparison between groups.

Statistical testing

Separate split-plot factorial ANOVA tests were performed on the DPOAE amplitude data and the CAP threshold data using treatment and sex as between subject variables and frequency as a repeated measure. In most instances, there were no sex differences and in those instances only the combined data are presented and the variable sex was dropped from the statistical analyses. For the analyses of DPOAE data, the range of frequencies analyzed was 5.2-16.9 kHz as this corresponds to the frequencies that are susceptible to NIHL from an octave band of noise centered at 8 kHz while eliminating the frequency range of approximately 20-25 kHz where instabilities occur in the DPOAE response due to outer ear canal resonance. A Greenhouse–Geiser correction was applied in all instances. Post hoc analyses were conducted using Bonferroni pair-wise multiple comparisons. Results obtained with a p value < 0.05 are reported as statistically significant.

RESULTS

The effects of noise treatment alone on auditory function and structural integrity of the cochlea four weeks following the noise exposure are portrayed in Figures 2-4. The distortion product test conducted 4 weeks post exposure showed a reduction in DPOAE amplitude, indicative of OHC impairment, in a dose-related manner within the anticipated frequency range predicted to show NIHL (see fig 2). The extent of the loss ranged from 10-20 dB within the frequency band for rats receiving 95 dB while the rats that received 85 dB noise treatment generally showed less than a 10 dB loss in the distortion product amplitude. The lowest noise treatment, 75 dB, yielded no noticeable shift in this functional measure. As there was no effect of sex upon extent of DPOAE

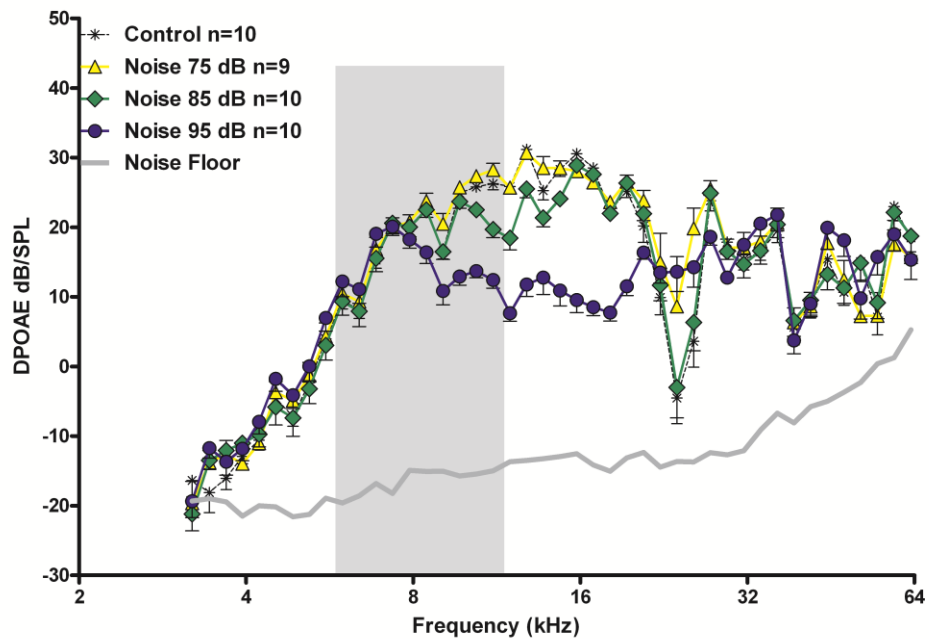


Figure 2: DPOAE amplitudes among rats exposed to noise treatment alone at 75, 85, and 95 dB(A) for 6 hr/day for 28 days compared to untreated control subjects. The shaded area denotes the range of frequencies contained in the noise exposure.

impairment, this factor was dropped from the final statistical analysis. The ANOVA test demonstrated a significant effect of noise treatment ($F_{3/35} = 13.68$, $p < .0001$), test frequency ($F_{17/595} = 239.57$, $p < .0001$), and a significant interaction term ($F_{51/595} = 21.94$, $P < .0001$). Bonferroni pairwise comparisons determined that the DPOAEs generated by rats exposed to 95 dB were significantly reduced relative to all other groups. However, there was no significant difference in DPOAE amplitude between control subjects and those exposed either to 75 dB or to 85 dB noise.

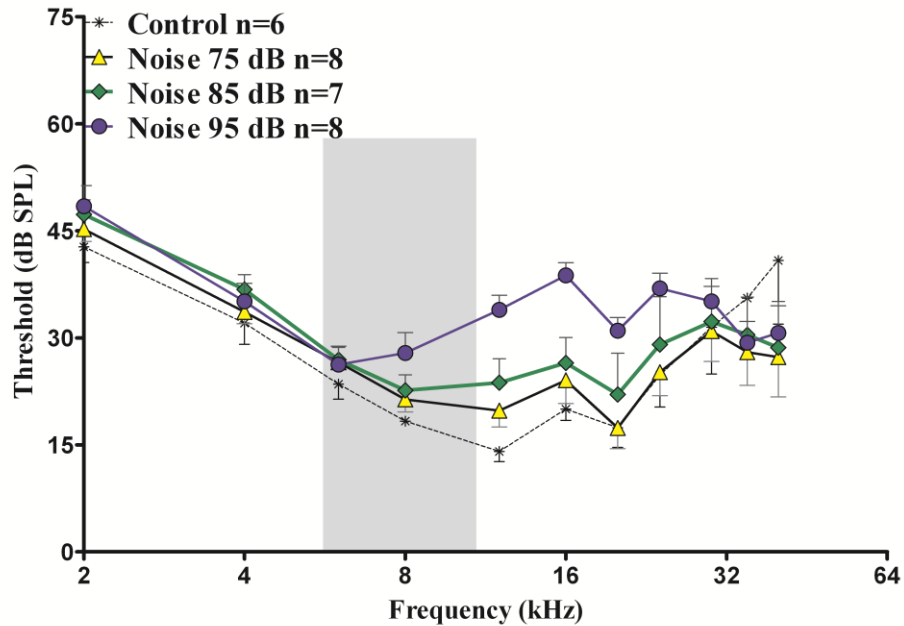


Figure 3: Auditory thresholds assessed 4 weeks following exposure of rats to noise treatment alone at 75, 85, and 95 dB(A) compared to untreated control subjects. The shaded area denotes the range of frequencies contained in the noise exposure.

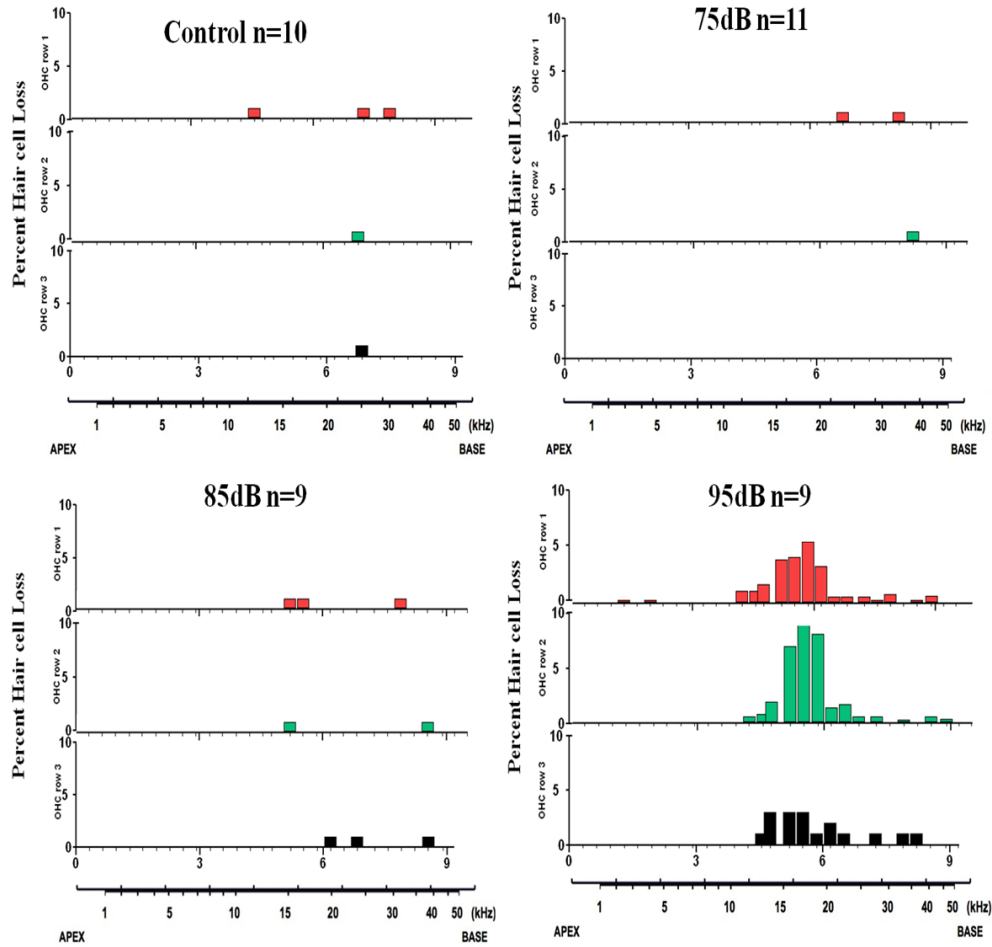


Figure 4: Cytocochleograms displaying hair cell death 4 weeks following exposure of rats to noise treatment alone at 75, 85, and 95 dB(A) compared to untreated control subjects.

The CAP test also conducted 4 weeks post exposure shows an elevation in auditory threshold among rats that received 95 dB of noise exposure (see fig 3). Within the frequency region of the noise exposure, threshold elevations of 15-20 dB were observed in these subjects. By contrast, rats receiving 85 dB of noise showed no more

than a 10 dB elevation of threshold relative to untreated controls and the rats receiving 75 dB showed only a 5 dB threshold elevation. The ANOVA conducted across treatment groups failed to show a statistically significant effect of noise intensity ($F_{3/25}= 1.36$, $p>.05$), but frequency ($F_{10/250}= 30.26$, $p<.0001$) and the noise intensity by frequency interaction ($F_{30/250}= 2.78$, $p <.0001$) did reach statistical significance. Based upon the significant interaction term, a step-down analysis that compared treatment groups within the frequency band (8-20 kHz), predicted to be affected by the octave band of noise was conducted. This analysis showed a significant effect of treatment ($F_{3/25}= 7.54$, $p< .001$), frequency ($F_{3/75}=10.39$, $p <.0001$) and a significant treatment by noise interaction ($F_{9/75}= 2.21$, $p <.05$). Bonferroni's multiple comparisons test identified a significant difference between control subjects and those exposed to 95 dB. The highest noise exposure group, 95 dB, also showed significantly poorer thresholds than either the 75 or 85 dB noise exposure group.

Figure 4 portrays the loss of OHCs caused by noise exposure as a function of location along the basilar membrane of the cochlea and, thereby, by sensitivity to tone frequency. Rats receiving 95 dB OBN noise exposure showed a highly selective loss of OHCs, but one limited to less than 10% within the 0.3 mm wide band that was used as the unit for counting. The loss was observed in all three rows of OHCs and occurred at locations corresponding to tone frequencies ranging from just under 15 kHz to 20 kHz. Rats receiving the two lower noise levels had sporadic hair cell loss that was indistinguishable from control subjects.

The effects of JP-8 exposure by itself are presented in figures 5-7. JP-8 exposure had no effect on DPOAE amplitude for either sex or for either the 10 day (data not

shown) or 4 week post exposure test. Figure 5 presents the DPOAE data for all subjects receiving JP-8 4 weeks after exposure. The data show practically no shift in DPOAE amplitude for any test frequency. Figure 6 portrays the effect of JP-8 exposure on auditory thresholds. Here there is no more than a 5 dB difference in auditory threshold among the groups with the largest difference from controls observed in the lowest JP-8

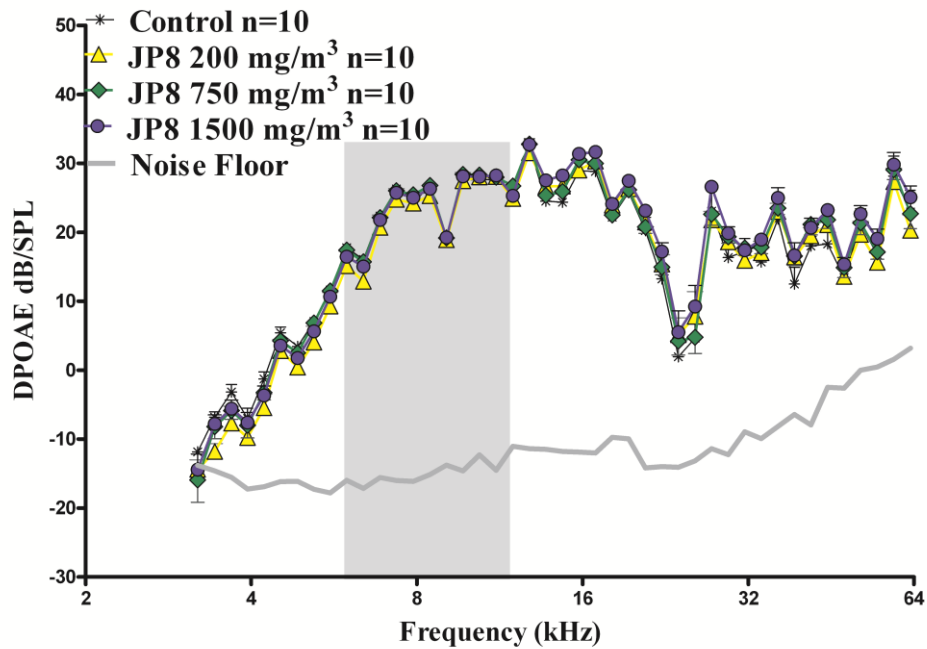


Figure 5: DPOAE amplitudes among rats exposed to 200, 750, and 1500 mg/m³ JP-8 jet fuel compared to untreated controls. The shaded area denotes the range of frequencies contained in the noise exposure.

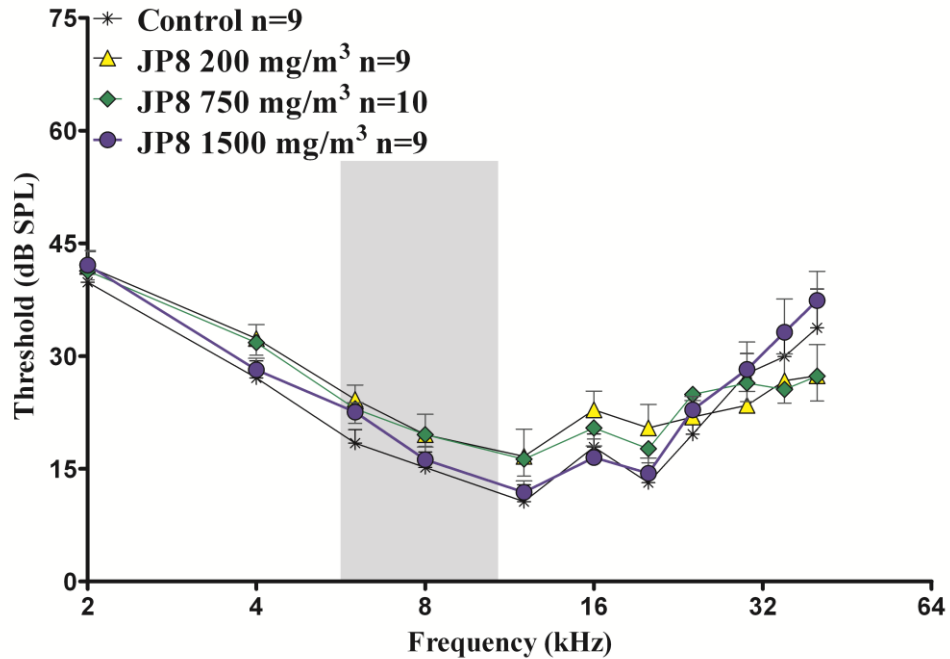


Figure 6: Auditory thresholds assessed 4 weeks following exposure of rats to 200, 750, and 1500 mg/m³ JP-8 jet fuel compared to untreated controls. The shaded area denotes the range of frequencies contained in the noise exposure.

exposure dose. Indeed, the highest JP-8 group resembles the control subjects more than the two other groups. Finally, figure 7 shows the loss of OHCs as a function of JP-8 exposure. There is no increase in hair cell loss among treated rats compared to controls.

Statistical analyses are consistent in establishing the equivalence of JP-8 treated rats and controls. In separate ANOVAs run on DPOAE and CAP data, the F values associated with treatment were smaller than 1.0 as were interactions that included the variable treatment.

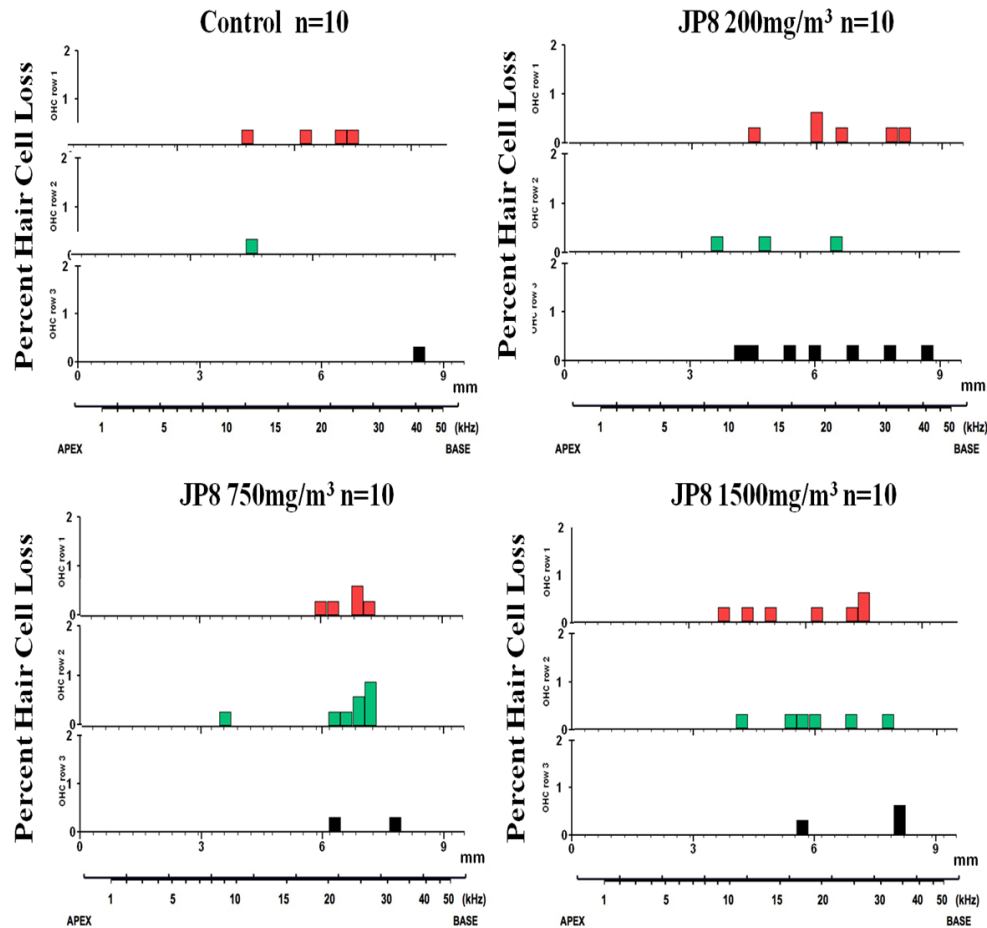


Figure 7: Cytocochleograms displaying hair cell death 4 weeks following exposure of rats to 200, 750, and 1500 mg/m³ JP-8 jet fuel compared to untreated controls.

The results of the combined continuous noise + JP-8 exposure study are presented in figures 8-11. Based upon the finding that 85 dB of noise produced minimal impairment of auditory function such that the cochlear function was indistinguishable statistically from controls (see fig 2 and 3) and that no cochlear histopathology was observed (fig 4),

this noise level was utilized in a parallel study with rats that were also being exposed to 200, 750, and 1500 mg/m³ JP-8 for 6 hr/day, 5 days/week for 28 days total.

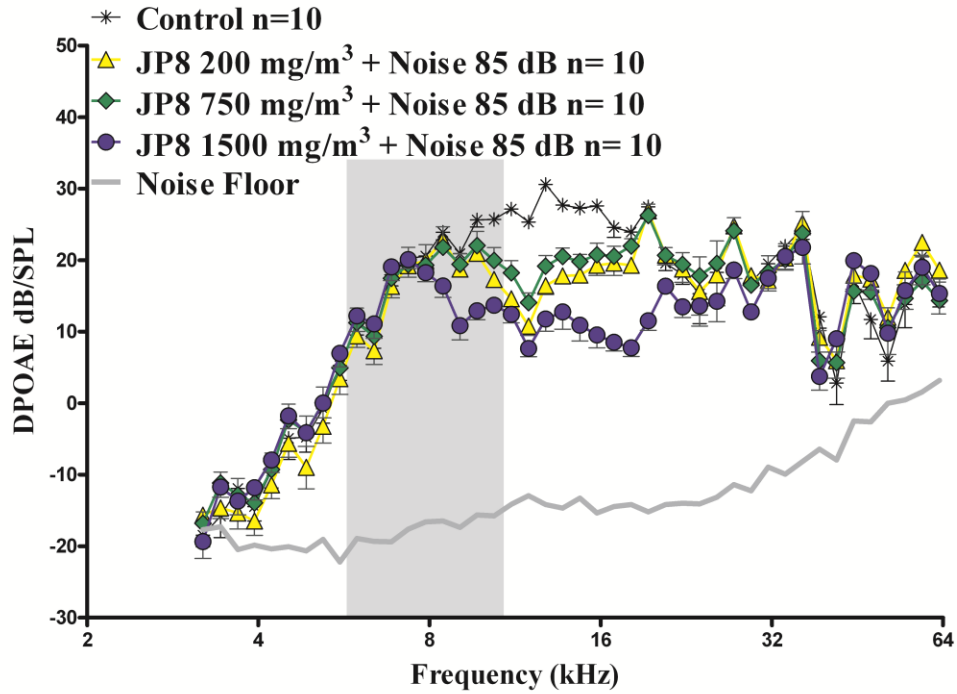


Figure 8: DPOAE amplitudes assessed 10 days after exposure of rats to continuous 85 dB OBN and 200, 750, and 1500 mg/m³ JP-8 jet fuel compared to untreated controls. The shaded area denotes the range of frequencies contained in the noise exposure.

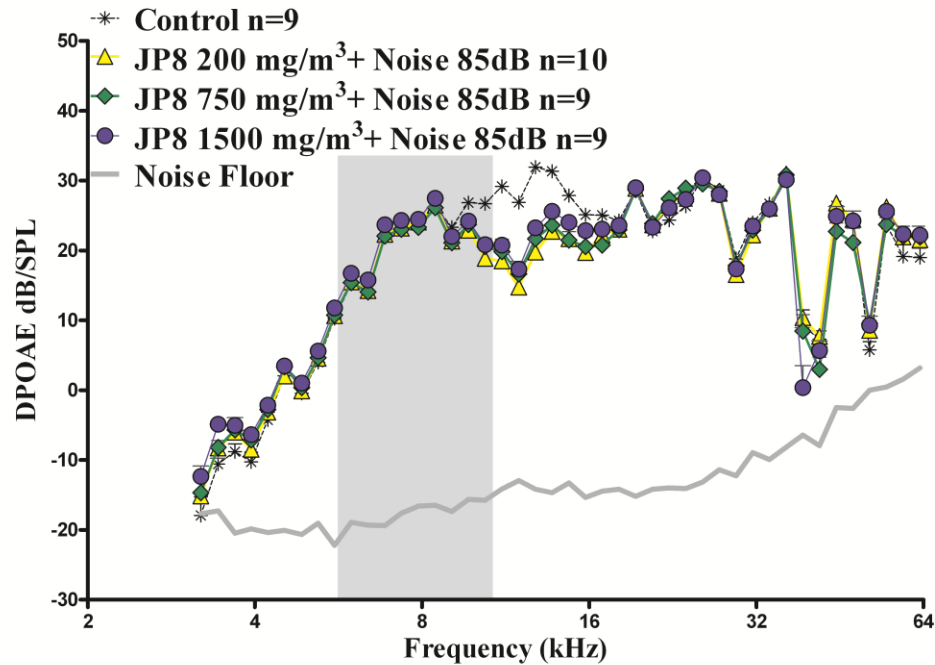


Figure 9: DPOAE amplitudes assessed 4 weeks after exposure of rats to continuous 85 dB OBN and 200, 750, and 1500 mg/m³ JP-8 jet fuel compared to untreated controls. The shaded area denotes the range of frequencies contained in the noise exposure.

At 10 days following combined JP-8 and noise exposure, marked impairment of DPOAE amplitude was observed relative to control subjects with the effect being particularly noticeable among the 1500 mg/m³ JP-8 + noise exposure group (see fig. 8). The impairment of the DPOAE response occurred at test frequencies that coincided roughly with the lower bound of the noise octave band and extended to about ½ octave above the upper bound of the octave band. A repeated measures ANOVA disclosed a significant effect of treatment ($F_{3/26} = 3.57$, $p < .03$), frequency ($F_{17/442} = 46.51$, $p < .0001$), and the

treatment by frequency interaction ($F_{51/442} = 6.90$, $p < .0001$). Neither the effect of sex nor sex by treatment was statistically significant (F 's < 1.0). Post hoc analysis by

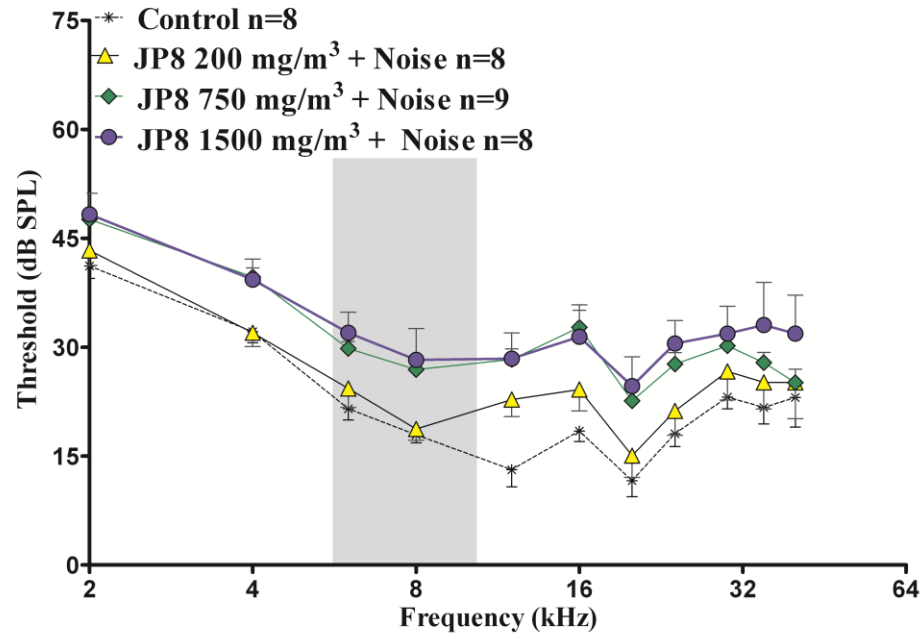


Figure 10: Auditory thresholds assessed 4 weeks following exposure of rats to continuous 85 dB OBN and 200, 750, and 1500 mg/m³ JP-8 jet fuel compared to untreated controls. The shaded area denotes the range of frequencies contained in the noise exposure.

Bonferroni's multiple comparisons test showed that the rats receiving the highest JP-8 exposure dose (1500mg/m³) + noise differed from controls. No other significant differences were found between treatment groups

Four weeks following the end of exposure, the extent and degree of DPOAE impairment was far more limited than at the 10 day time point (see fig 9). However, a reproducible decrease in the DPOAE response was still observed. Notably, all of the JP-8

+ noise groups were impaired relative to control subjects but did not differ from each other.

The ANOVA documented a significant effect of treatment ($F_{3/33}=10.62$, $p<.0001$) frequency ($F_{17/561}= 428.87$, $p <.0001$) and the treatment by frequency interaction ($F_{51/561}= 15.48$, $p<.0001$). Each of the fuel + noise groups showed significantly lower DPOAE responses than the control group. The three JP-8+ noise dose groups did not vary significantly among themselves based upon Bonferroni's pairwise comparisons.

Pure tone auditory thresholds were elevated in the JP-8 + noise rats relative to control subjects (see fig 10). In this instance, the 1500 mg/m^3 JP-8 exposed rats showed the largest impairment although the 750 mg/m^3 JP-8 + noise subjects showed similar impairment over a more limited range of frequencies. The auditory thresholds of 200 mg/m^3 JP-8 exposure + noise subjects were quite similar to that of controls. The ANOVA showed a significant effect of treatment ($F_{4/29}= 2.72$, $p <.05$) with Bonferroni comparisons identifying a reliable difference only between the control group and the group exposed to 1500 mg/m^3 JP-8 + noise. Sex was also significant ($F_{1/29}= 4.89$, $p < .05$) with males having poorer hearing than females across all treatment groups. Frequency was also significant ($F_{10/290}= 70.21$, $p < .0001$) although none of the interactions of treatment with sex or with frequency were significant (F 's < 1.0).

There was sporadic OHC loss among rats exposed to the lowest two doses of JP-8 + noise equivalent to that seen in the control subjects (see fig. 11). However, rats that received 1500 mg/m^3 JP-8 exposure + noise demonstrated a consistent loss of OHCs that was restricted to the middle turn of the cochlea roughly between 4.5 and 6.5 mm from the apex. This area corresponds to a region that encodes frequencies from about 12-20 kHz.

The loss was approximately 3 % in the 3rd row of outer hair cells, 8% in the second row of outer hair cells, and 4% in the 1st row of outer hair cells.

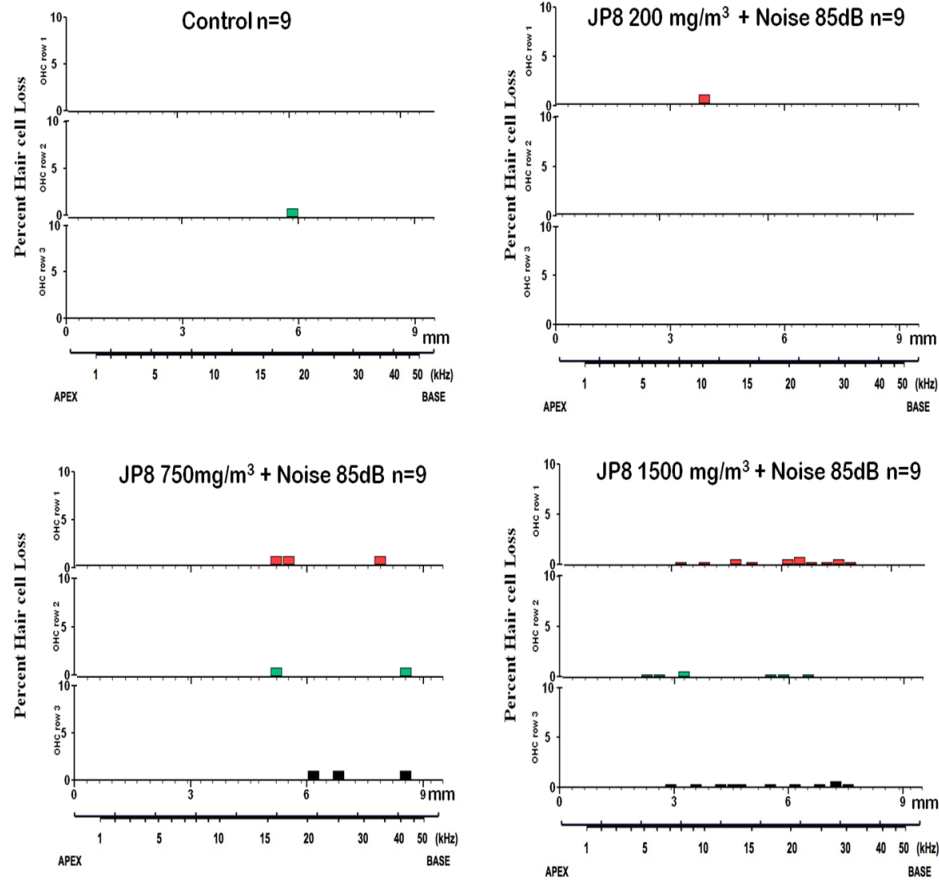


Figure 11: Cytocochleograms displaying hair cell death 4 weeks following exposure of rats to 200, 750, and 1500 mg/m³ JP-8 jet fuel + noise compared to untreated controls.

The effect of JP-8 exposure on the auditory system in rats exposed to intermittent noise exposure is presented in fig 12-14. At 10 days following exposure, a marked impairment of DPOAE amplitude was observed among rats of both sexes exposed to noise and those receiving JP-8 + noise (see fig 12a and b). The impairment of the

DPOAE response occurred at test frequencies that coincided roughly with the lower bound of the noise octave band and extended to about ½ octave above the upper bound of the octave band. Four weeks following the end of exposure, the extent and severity of DPOAE impairment is far more limited in both sexes than at the 10 day time point (data not shown). However, a reproducible decrease in the DPOAE response was still observed among both the rats that received JP-8 + noise and those exposed to noise alone. This finding held true for both the male and female rats. The ANOVA conducted on the DPOAE data at 10 days post exposure showed significant effects of treatment ($F_{3/36} = 37.98$, $p < .0001$), frequency ($F_{17/612} = 52.37$, $p < .0001$) and the treatment by frequency interaction ($F_{51/612} = 24.29$, $p < .0001$). Bonferroni pairwise comparisons showed that both the noise alone and the noise + JP-8 groups were significantly impaired relative to the control and JP-8 only rats. However, the two noise groups were not different statistically. Similar findings were identified 4 weeks after exposure with treatment ($F_{3/36} = 36.44$, $p < .0001$) frequency ($F_{17/612} = 45.28$, $p < .0001$), and the interaction of these terms ($F_{51/612} = 26.24$, $p < .0001$) all meeting statistical significance. And as was true at 10 days post exposure the two noise groups differed significantly from both controls and JP-8 alone, but did not differ from each other.

Pure tone auditory thresholds were significantly impaired in the JP-8 + noise rats relative to all other groups (see fig 13). The effect was particularly noticeable at high test frequencies beyond those that would be expected to occur due to noise alone. The effect stems from a profound disruption of threshold in the male rats that received JP-8 + noise while female rats that received the combined exposure do not show greater impairment of

hearing than the noise only rats. A significant concern, however, is that two male rats that had received combined treatment could not be tested. In one instance, damage to a major

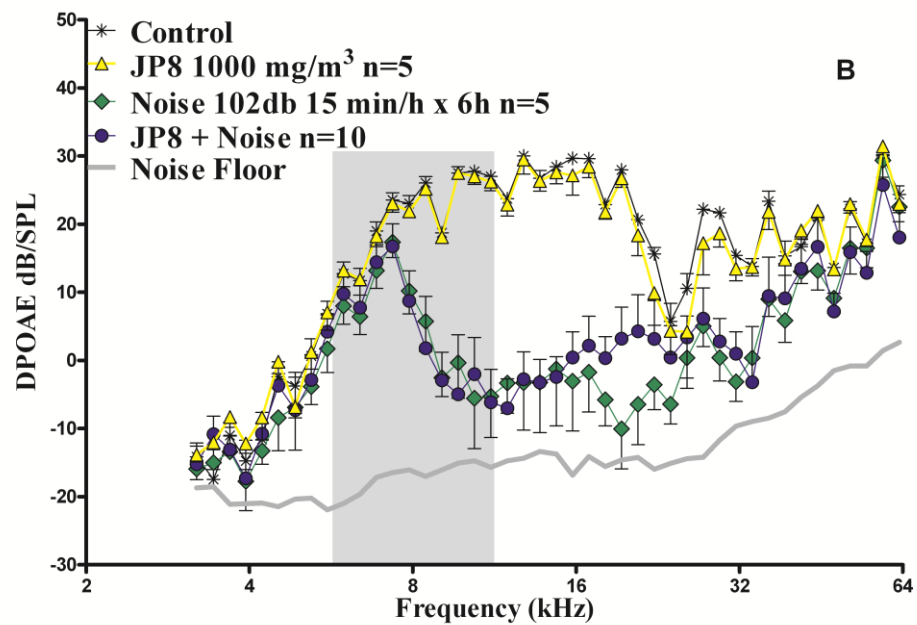
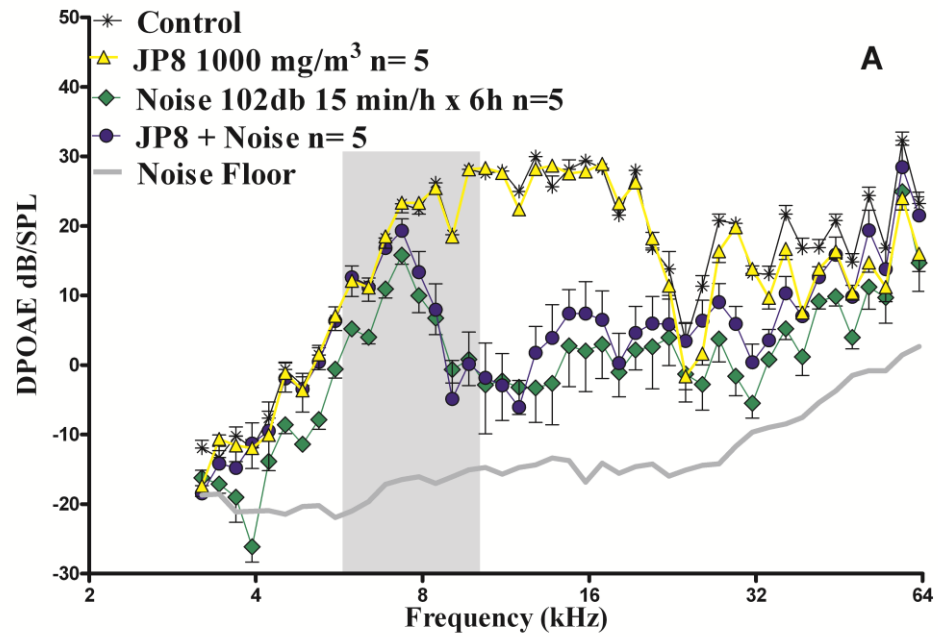


Figure 12: DPOAE amplitudes 10 days post exposure among female (A) and male (B) rats exposed to 1000 mg/m³ JP-8 + intermittent noise of 102 dB(A) for 6 hrs/day for 28 days. Noise was turned on for 15 min out of each hour for a total of 90 min exposure. Also portrayed are rats receiving either JP-8 alone, noise alone, and control subjects.

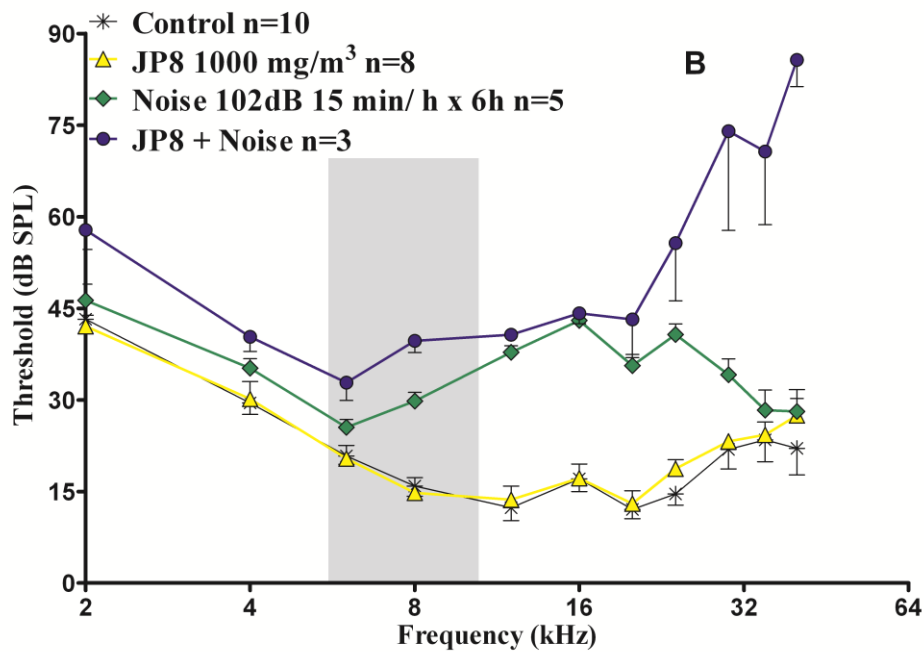
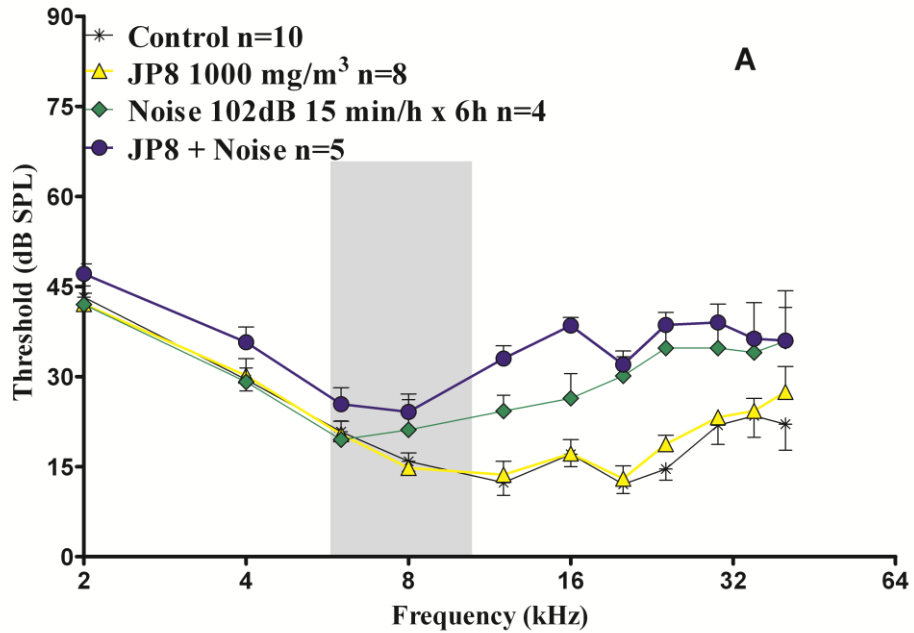


Figure 13: Auditory thresholds among female (A) and male (B) rats exposed to 1000 mg/m³ JP-8 + intermittent noise of 102 dB(A) for 6 hrs/day for 28 days. Noise was turned on for 15 min out of each hour for a total of 90 min exposure. Also portrayed are rats receiving either JP-8 alone, noise alone, and control subjects.

artery occurred during surgery leading to the death of the rat. In the other case, the round window was punctured in the process of placing an electrode onto this structure.

Consequently, the CAP thresholds for the male combined exposure subjects reflect the effects seen in only 3 rats while the DPOAE and the histopathology are based upon all 5 male rats. Analysis of auditory thresholds showed a significant effect of treatment ($F_{3/28} = 20.79$, $p < .0001$), sex ($F_{1/28} = 6.01$, $p < .03$), and frequency ($F_{10/280} = 35.92$, $p < .0001$) main effects. The treatment by sex interaction did not meet statistical significance. Bonferroni multiple-comparison test showed a significant difference between rats receiving Noise + JP-8 and all other treatment groups including noise alone. In addition, the noise only rats showed significantly elevated auditory thresholds relative to control subjects.

The effects of experimental treatment on the cochlea were also assessed by counting the number of missing/dead hair cells at the time of CAP threshold testing (see fig 14). As in all other studies we have conducted, the loss of OHCs was sporadic and under 1% both in the control subjects and in the rats that received JP-8 alone. There was no difference in OHC death between these two groups. Among those rats that received noise treatment alone, OHC loss tended to occur at locations of the cochlea that are most sensitive to sound frequencies between approximately 10-30 kHz. Also, the extent of

noise-induced hair cell death tended to be somewhat greater among male rats than among female rats. Finally, among male rats, those that received combined exposure to JP-8 + noise showed a broader region with missing hair cells than the noise only male rats and tended to have somewhat greater rates of hair cell loss as well. For female rats, the subjects receiving the combination of JP-8 and noise actually showed less hair cell loss than did the subjects exposed to noise alone.

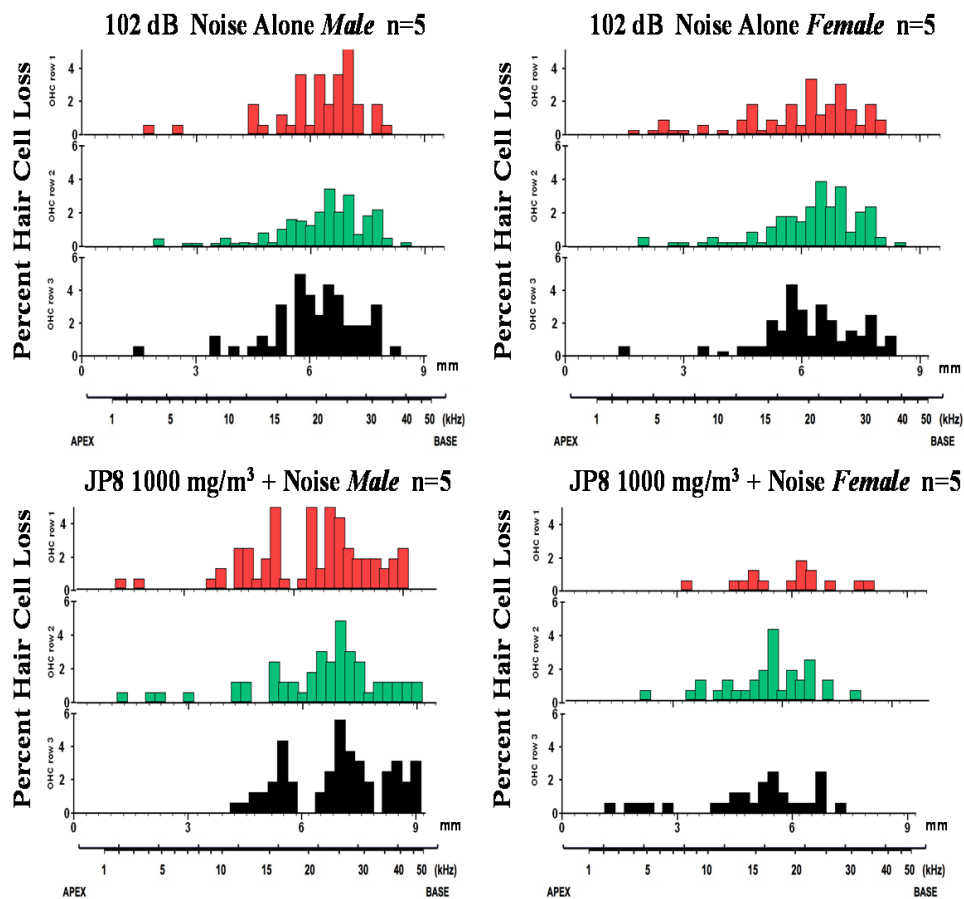


Figure 14: Cytocochleograms depicting the loss of OHC among rats exposed to 1000 mg/m³ JP-8 + intermittent noise of 102 dB(A) for 6 hrs/day for 28 days. Noise was turned

on for 15 min out of each hour for a total of 90 min exposure. Also portrayed are rats receiving either JP-8 alone, noise alone, and control subjects

DISCUSSION

These experiments have focused on the vulnerability of auditory function and of cochlear integrity to exposure from JP-8 jet fuel with and without simultaneous noise exposure. The results demonstrate that JP-8 by itself is unable to disrupt cochlear function as reflected in the DPOAE, the auditory threshold or damage to OHCs even at a dose of 1500 mg/m^3 for 6 h/day, roughly 7.5 times the permissible human exposure level on a TWA basis. Similarly, the noise intensity used in combination with the JP-8 exposure, 85 dB for 6 hr/day, has no significant functional or histopathological consequences. Yet when this moderate noise exposure is combined with simultaneous JP-8 jet fuel exposure, rats that received 1500 mg/m^3 of that fuel showed permanent impairment of the DPOAE response, an elevation in the CAP, and discrete lesion of OHCs at a cochlear locus consistent with the functional impairment. While a loss of only 10% of these hair cells must be considered to be a small effect, its reproducibility across subjects provides strong evidence to bolster the functional impairments observed.

Though it is clear that only the highest JP-8 dose yielded a sufficiently large impairment in combination with noise to produce a reliable statistical difference there is evidence of a trend toward impaired function in subjects exposed to 750 mg/m^3 JP-8 + noise. The LOAEL for JP-8, 1500 mg/m^3 for 6 hr/day, is 7.5 times the permissible human exposure level and 750 mg/m^3 JP-8 is roughly 4 times greater than the PEL. This finding that JP-8 + Noise exposure significantly impairs the cochlea relative to rats

exposed to noise alone replicates the results of a similar study conducted in this laboratory using 1000 mg/m³ JP-8 and a higher noise exposure level (105 dB) over a 4 hr/day, 5 day exposure period (Fechter et al., 2007).

The results obtained using intermittent noise of 102 dB along with JP-8 are somewhat less clear because this noise exposure level by itself produced substantial impairment of OHC function as reflected by DPOAE amplitude reduction and OHC death. Moreover, combined exposure to JP-8 + noise did not yield an increased loss in OHC function and structure than did noise alone. However, when the neural output of the cochlea is considered, the male rats that receiving JP-8 (1000 mg/m³) + intermittent noise, showed a far greater elevation of auditory thresholds than do rats that receive noise exposure alone. This elevation was seen not only in the frequency range that would be anticipated to be affected by the OBN selected, but also at higher test frequencies. The spread of impairment by chemical contaminants presented along with noise has been previously observed in the case of subacute JP-8 + noise exposure (Fechter et al., 2010).

Another feature of the enhanced susceptibility to noise observed with simultaneous JP-8 exposure is the finding that the CAP response is disrupted more reliably than is the DPOAE response. The CAP monitors the production of synchronous auditory nerve activity at the inner hair cell-spiral ganglion cell synapse while the DPOAE response monitors OHC function. While CAP threshold sensitivity can certainly be degraded by impairment of OHCs in as much as the OHCs serve as a “gain control” for the inner hair cells, the neural elements, the current evidence suggests that the inner hair cells and spiral ganglion cells may be impaired directly by JP-8 + noise.

It is not obvious why male rats appear to be more vulnerable to the enhancement of NIHL by JP-8 jet fuel exposure. However, the finding of enhanced male susceptibility was found not only in the final experiment where intermittent noise was presented along with JP-8 jet fuel, but also in terms of auditory threshold specifically regardless of group treatment when continuous noise was paired with three different doses of JP-8. While the enhanced sensitivity of male rats to JP-8 + noise might reflect a true sex difference in terms of vulnerability to noise, for example, it is also possible that it might reflect toxicokinetic factors related to body fat storage rather than to a sexual dimorphism. The male and female Fischer 344 rats in our studies showed distinctly different patterns of weight gain. On average, female subjects had average weights of 148 at the beginning of exposure and averaged 165 g at the end of the 4 week exposure. During the same time period males initially averaged 187g and averaged 243 g at the end of exposure. It is possible that the difference in body fat levels between the sexes resulted in greater storage of the JP-8 fuel in male rats and, thereby, longer periods of elevated JP-8 body burdens.

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